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Presence of recombination hotspots throughout *SLC6A3*

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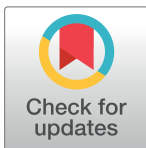

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Abstract

The human dopamine transporter gene *SLC6A3* is involved in substance use disorders (SUDs) among many other common neuropsychiatric illnesses but allelic association results including those with its classic genetic markers 3'VNTR or Int8VNTR remain mixed and unexplainable. To better understand the genetics for reproducible association signals, we report the presence of recombination hotspots based on sequencing of the entire 5' promoter regions in two small SUDs cohorts, 30 African Americans (AAs) and 30 European Americans (EAs). Recombination rate was the highest near the transcription start site (TSS) in both cohorts. In addition, each cohort carried 57 different promoter haplotypes out of 60 and no haplotypes were shared between the two ethnicities. A quarter of the haplotypes evolved in an ethnicity-specific manner. Finally, analysis of five hundred subjects of European ancestry, from the 1000 Genome Project, confirmed the promoter recombination hotspots and also revealed several additional ones in non-coding regions only. These findings provide an explanation for the mixed results as well as guidance for selection of effective markers to be used in next generation association validation (NGAV), facilitating the delineation of pathogenic variation in this critical neuropsychiatric gene.

Introduction

By sequestering dopamine (DA) into presynaptic neurons, the dopamine transporter (DAT) regulates spatio-temporal components of DA transmission. As a critical regulator of DA transmission, DAT contributes to voluntary movement, reward and mnemonic functions of the brain and modulates the efficacy of therapeutic drugs targeted to this plasma membrane protein. DAT expression is highly circumscribed in discrete regions throughout the brain and the expression of the human DAT gene (*SLC6A3*) varies among subjects[1–5]. Thus DNA sequence variations in the regulatory regions specially the promoter of *SLC6A3* may contribute to altered expressional patterns in the brain, dopamine-related individuality as well as diseases [6, 7]. The essential roles of DAT in brain function have mandated extensive studies of *SLC6A3* associations with behaviors and diseases.

During the last twenty five years *SLC6A3* has been extensively studied for genetic associations but the association studies with different markers located in *SLC6A3*'s 3' regions obtained mixed results. On chromosome 5 (chr5), *SLC6A3* spans 70 kilobase (kb) from the 5' promoter to 3'untranslated region (3'UTR, located in Exon 15). Sporadic genetic markers in several regulatory regions throughout *SLC6A3*, including the 5' promoter, Intron 8 and 3'UTR, have been used in hundreds of association studies on more than eight different diseases and a number of human behaviors. Vast majority of these studies used a classical variable number tandem repeat located in the 3'UTR (3'VNTR/rs28363170) and another more recently used VNTR in Intron 8 (Int8VNTR/rs3836790)[8, 9]. As a result, findings from these association studies, especially those with 3'VNTR, in various populations were largely inconclusive or showed small effect sizes, such as studies on schizophrenia and bipolar disorder among others [10–15]. In particular, *SLC6A3* has been well implicated in the etiology and treatment of attention deficit hyperactivity disorder (ADHD)[16–22] but human genetic association studies with the 3'VNTR could not obtain consistent positive signals[23–25]. Another example was pharmacogenetics of Parkinson's disease (PD): 3'VNTR or In8VNTR was associated with differential response to pharmacotherapy of PD[26–30] but not based on other studies [31, 32]. Moreover, *SLC6A3* genotype was found to modulate the risk of pesticide exposure for PD by some studies [33]but not by others[34]. The lack of evidence-based selection of markers resulted in the mixture, unfortunately causing little motivation to add more association studies with any markers in the *SLC6A3* genetic field. Importantly, the unreliable human genetic findings are inconsistent with ample positively-related evidence for *SLC6A3* activity *versus* phenotypes from other approaches such as pharmacology, imaging and animal genetics[20, 35–39].

In contrast to 3'VNTR, its promoter markers were more consistently associated with ADHD in various populations [40–47]. Consistently, we and others have shown that the 5' promoter regions display varying regulatory activity and also in a haplotype-dependent manner *in vitro*[48–51]. Findings from rodent genetic studies have demonstrated the causality of reduced DAT activity on various phenotypes[37, 52–56]. These findings suggest that polymorphism- or haplotype-dependent *SLC6A3* promoter activity may confer risk for related diseases and that genetic association studies should have resulted in consistent positive findings.

One explanation for the current elusiveness of the association findings with the 3'VNTR was that this marker is far away from upstream regulatory regions, including Int8VNTR and the 50 kb-away 5' promoter, and unable to capture the related information due to high recombination rates or weak linkage disequilibrium (LD). Other explanation is that different populations carry different frequencies of the same markers or even different disease loci. In either case, these already used genetic markers were unlikely the underlying disease loci. *In vitro* studies have shown polymorphisms in the 5' core promoter, Int8VNTR and 3'VNTR all regulated promoter activity[49, 57–61]. This information suggests that it be critical for association studies to use genetic markers in all distinct regulatory regions, in order to capture variable *SLC6A3* expression as a whole and identify the underlying haplotypes and signaling pathways [51].

To clarify these possibilities, it is necessary to deeply sequence the regulatory regions for a better understanding of the *SLC6A3* genetic structure including the 3' VNTRs, given the implications of *SLC6A3* in a spectrum of diseases and other behavioral characteristics. This task requires systemic discovery of polymorphisms and haplotypes in the regulatory regions, through targeted deep-sequencing that is helpful in discovery of novel functional loci or mutations in different fields[62–69]. In other words, the presence of multiple *SLC6A3* regulatory regions mandates mechanistic studies of the *SLC6A3* genetics, to help delineating functionally distinct *SLC6A3* haplotypes.

In this targeted deep-sequencing study, we uncover unique and common polymorphisms and haplotypes, and recombination hotspots for two major U.S. populations, African Americans (AAs) and European Americans (EAs), for finding generalization. The findings may help explain the mixed association findings[25, 70, 71] and instruct our future strategy for identifying disease loci in *SLC6A3*[29, 72, 73].

Materials and methods

Subjects

Sixty unrelated subjects were selected from the Collaborative Studies on Genetics of Alcoholism (COGA) pedigrees[74] and their de-identified genomic DNAs were provided by COGA through the Coriell Institute (NJ, U.S.A.) with the approval of National Institute on Alcohol Abuse and Alcoholism (NIAAA). Subjects gave their informed consent to the COGA study. From each of the COGA pedigrees, we selected the grandparents and their offspring's spouses that came from outside the pedigree as unrelated subjects and the unrelatedness was verified by genomic control[75]. They included two cohorts: 30 AAs and 30 EAs. Each cohort consisted of 15 controls and 15 patients with substance use disorders (SUDs) (see Table 1). The 30 control subjects were all unaffected based upon the Diagnostic and Statistical Manual of Mental Disorders III Revision (DSM-IIIR), Feighner Criteria and International Classification of Diseases, Tenth Revision (ICD-10). All of the 30 affected subjects met at least two of the DSM-IIIR, Feighner Criteria and ICD-10 criteria for alcohol dependence. This study was approved by McLean Hospital Institutional Review Board.

DNA sequencing

A two-step "boost/nest" polymerase chain reaction (PCR) strategy was used to sequence the 18 kb promoter regions at Polymorphic DNA Technologies, Inc (Alameda, CA). We first did a boost reaction for a larger PCR amplicon and then used this amplicon as a template for the nest reaction, followed by sequencing of the nest product. The conditions for the boost PCR reaction were identical to the nest with the following exceptions: 10 ng of genomic DNA was used for the boost, then 1 μL of boost product as template for the nest reaction. The two reactions used two different pairs of different primers. PCR cycle was: 94˚C for 4 min, 25 cycles of 94˚C for 20 sec, 55˚C for 25 sec and 72˚C for 1 min, followed by an extension of 72˚C for 7 min. Double-stranded DNA sequencing was carried out by using the Applied Biosystems 3730/3730*xl* chemistry in a 384-well format.

Genotyping

A multiplex PCR-restriction fragment length polymorphism (RFLP) method was used for VNTR genotyping. PCR followed standard protocols, with Fast PCR Master Mix (Fermentas, Glen Burnie, MD, U.S.A.) as described before[76]. For -14kb-VNTR, the PCR product was 913-bp, 733-bp, 652-bp, and 771-bp for 1-, 2-, 3-, and 4-repeat respectively. 5'VNTR was

Table 1. Demographic information on 60 COGA subjects used.

	AA (30)			EA (30)		
	Control (15)	Case (15)	Total	Control (15)	Case (15)	Total
Male	7	7	14	8	8	16
Female	8	8	16	7	7	14
Average age	40.4 ± 4.3	44.3 ±3.6		49.0 ± 3.8	48.1 ±3.6	
Age range	21–72	27–70		24–77	24–63	

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426-bp, 486-bp, 546-bp, and 606-bp for 6-, 7-, 8-, and 9-repeat respectively. Int8VNTR was 291-bp and 321-bp for 5- and 6-repeat, and 3'VNTR was 441-bp, 481-bp and 521-bp for 9-, 10- and 11-repeat. These VNTR's PCR products were subject to agarose electrophoresis directly. The allele sequences of -10kb-pA (polyadenine) and simple sequence length polymorphism (SSLP) were verified by TA cloning (Invitrogen, Carlsbad, CA, U.S.A.) and DNA sequencing of TA clones (five to six per subject for a 95% confidence on biallelic polymorphisms). All Chromas sequencing graphs were refereed manually by two researchers independently for double-verification of sequence accuracy. All primers used are listed in S1 Table.

Genetic analysis

Linkage disequilibrium (LD), expressed as D' and r^2[77], was analyzed by using Haploview (http://www.broadinstitute.org/haploview/haploview) for biallelic polymorphisms and SHEsis (http://analysis.bio-x.cn/myAnalysis.php) for multiallelic polymorphism[78, 79]. Haplotyper (http://www.people.fas.harvard.edu/~junliu/Haplo/click.html) and PHASE (http://www.stat.washington.edu/stephens/phase/download.2.0.2.html) softwares were used for haplotype inference[80]. Recombination fraction was estimated by LDhat (http://www.cecalc.ula.ve/BIOINFO/servicios/herr1/LDhat/readme.html).

To evaluate general chromosomal recombination, five populations of European ancestry in the 1000 Genomes Project (1KGP)[81, 82], including US Caucasians, Great Britain, Italy, Spain, and Finland, were combined for reliability to reveal recombination hotspots in *SLC6A3* by using the published FastEPRR protocol[83].

To localize genome wide association study (GWAS) markers in this gene, three SUDs GWAS datasets, all past their embargo periods, were downloaded from the dbGaP,[84] including Collaborative Study on the Genetics of Alcoholism [85] (COGA, phs000125.v1.p1), Study of Addiction: Genetics and Environment (SAGE, phs000092.v1.p1) and the Australian twin-family study of alcohol use disorder (OZALC, phs000181.v1.p1). Datasets were cleaned or quality-controlled extensively by using a published protocol,[86] followed by imputation as described before.[87] Basic manipulations of datasets used PLINK[88].

To estimate Tajima's D statistic in 30 unrelated COGA subjects, we calculated nucleotide diversity θ as the number of segregating sites, S, divided by a_1, where $a_1 = \sum_{i=1}^{59} \frac{1}{i} = 4.6632$, divided by the number of nucleotides sequenced. Heterogeneity π was estimated by $k = \sum_{j=1}^{S} 2p_j(1 - p_j)$, divided by 1–(1/59) = 0.983051, divided by the number of nucleotides sequenced, where p_j was the observed frequency of the jth diallelic polymorphism. Statistic D was calculated using the θ and π, according to Tajima[89]. Phylogenic analysis was carried out by ClustalX (http://www.bioinformatics.ubc.ca/resources/tools/index.php?name=clustalx), with neighbor joining method for clustering and phylogenetic tree was displayed by TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) for cladistic analysis[90, 91]. Validations used MEGA7[92] under the Kimura 2 parameter with Gamma Distributed as rates among sites model for nucleotide substitutions. The relatedness results were shown in a radiation graphic, rather than in traditional trees, for better visualization.

Results

Unique variation and ethnic differences

We sequenced the 18 kb promoter region including the 16 kb 5' region, Exon 1, and the 2 kb Intron 1 and further genotyped the two VNTRs in Intron 8 and 3'UTR among 30 AAs and 30

EAs. A sample size of 30 allowed 80% confidence of detecting an allele with a frequency of 2.6% or 95% confidence of detecting an allele with a frequency of 4.9%. Based on more than 7,300 PCR reactions and sequencing of more than 2.4 mega-bases (Mb), the 60 subjects were found to carry 134 polymorphisms in the 18 kb 5' promoter regions and 20.1% of them were novel (S2 Table). The 5' promoter had two VNTRs, one novel at -14280 (-14kb-VNTR) and another at -11000 (5'VNTR/rs70957367); a novel -15.0kb-indel (insertion/deletion), one novel variable poly A at -10331 (-10kb-pA) and one novel SSLP at +1531 (use of the first base of Exon 1 as +1 and negative numbers for upstream of the promoter). All of these polymorphisms were all confirmed by TA cloning and DNA re-sequencing. The novel -14kb-VNTR had four alleles that were formed by multiple indels, di-nucleotide polymorphisms (DNPs) and single nucleotide polymorphisms (SNPs) (see S1 Fig *upper panel*). 5'VNTR had 7–9 repeats of imperfect 60 bp, whose primary sequence was reported previously[76]. -10kb-pA had 9–11 As. SSLP in Intron 1 had nine different length polymorphisms (S1 Fig *lower panel*). Int8VNTR had two alleles, 5 and 6 repeats of 30 bp and 3'VNTR had 9–11 repeats of 40 bp as previously discovered[8, 60, 93, 94].

For the sample size of 30 subjects, the AA group had 108 promoter polymorphisms and the EA group, 79 polymorphisms (see Fig 1A for distribution). The polymorphism density was 6.1/kb in AA and 4.5/kb in EA. Between AA and EA, 53 of the 134 polymorphisms were shared; the former carried 55 additional unique polymorphisms and the later carried 26 additional EA-specific ones. +24G/T (rs45611137) was the only Exon 1 mutant (minor allele frequency (MAF): 0.0167), present in the AA, not in the EA cohort.

Recombination rate (ρ)

The core promoter region at -2.3 kb and Intron 1 at +1.6 kb both displayed higher recombination rates than upstream promoter regions (Fig 1B). Two other hot spots were at -10.4 kb and -12.6 kb. In the core promoter region, ρ was 3.57 in AA and 2.55 in EA, representing the hottest region for recombination in the *SLC6A3* promoter. This rate decreased sharply as the distance from the -2.3 kb spot increased towards either side (see *Insert* in Fig 1B). In this core promoter region, the average ρ value was 1.48 in the AA cohort, 1.4-fold higher of the ρ value in the EA cohort. The Intron 1 had another hotspot. The largest difference in recombination rate between the two cohorts was three-fold at -15.7 kb, the 5' end of the promoter regions where ρ value was 1.29 in the AA and 0.43 in the EA cohort. The average ρ value was 1.26 in the AA, 1.3-fold higher of the ρ value in the EA cohort. Although we have not stratified the analysis by sex, the findings persistently pointed to four recombination hotspots in the *SLC6A3* promoter of the COGA cohorts.

LD

The overall AA LD was low across the entire regulatory regions (average D' = 0.8214 and square of the Pearson correlation coefficients $r^2 = 0.1283$). There was a weak 6 kb block from -6234A/G (rs1354139) to -68T/A (rs2975226) (D' = 0.8487 and $r^2 = 0.1621$, see Fig 1C *left panel*). Intron 1 displayed relatively weak LD within the Intron (D' = 0.8414 and $r^2 = 0.0964$). In particular, the 5' end of Intron 1 from +24G/T (rs45611137) to +579G/A (rs28382214) represented a major subregion of weak LD (D' 0.8938 and r^2 0.0438). Int8VNTR displayed low LD (D' 0.6126 and r^2 0.06524) with the upstream polymorphisms. Int8VNTR had perfect LD with 1787G/A (rs11564757) (D' and r^2 both = 1) and high LD with -15.0kb-indel, -10250C/T (rs72717506), -9701C/T (rs10063727), -4913A/G (rs10079467), -1675T/C (rs11564751) and -1479G/T (rs6413429) (D' = 1 and $r^2 = 0.4$).

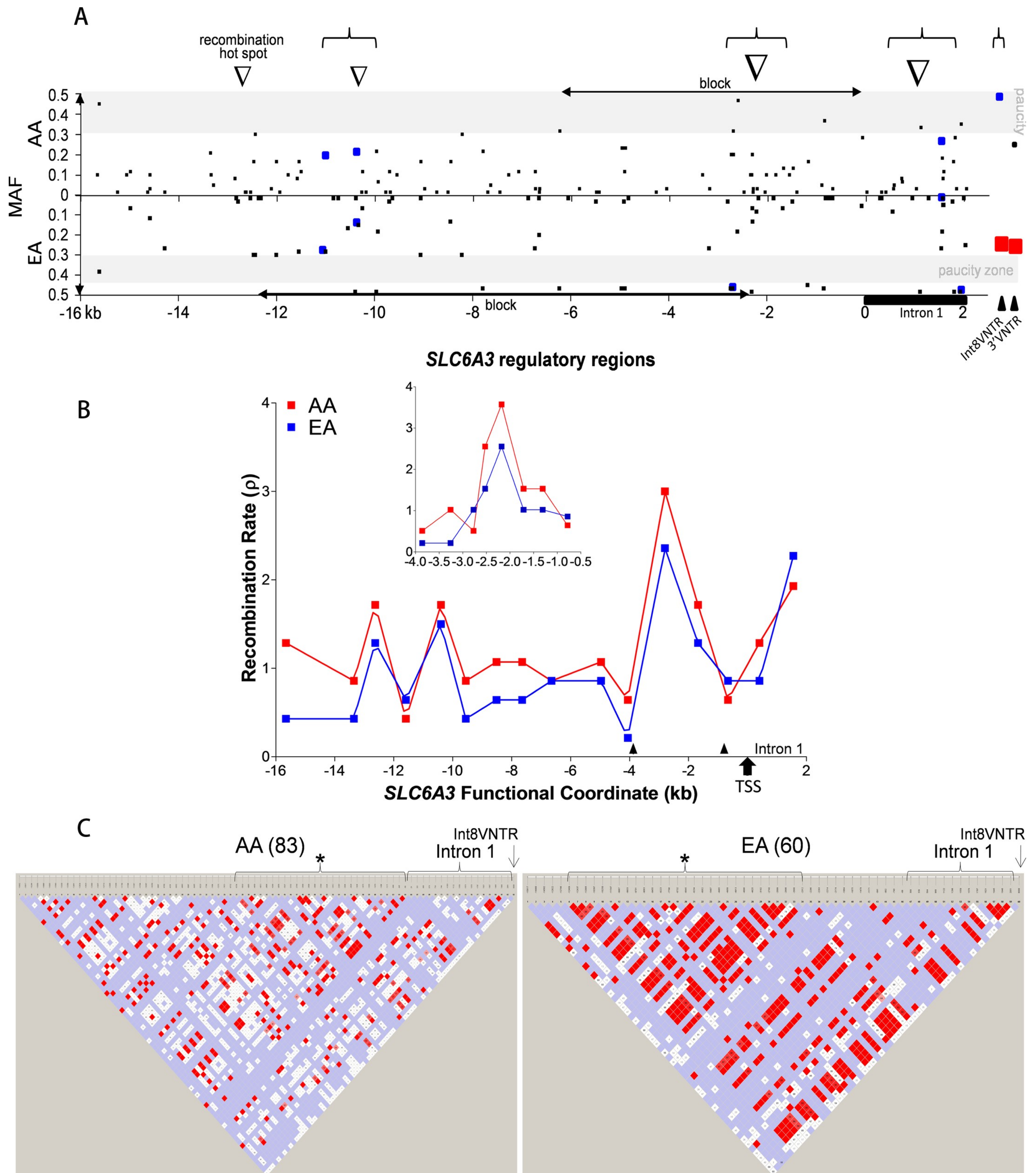

Fig 1. *SLC6A3* regulatory region polymorphisms. (A) Asymmetric distribution of common polymorphisms between the AA (*upper panel*) and the EA (*lower panel*) cohorts. Each polymorphism is indicated by small black square. Gray areas indicate paucity of polymorphisms for indicated MAF range. Black horizontal bar, location

of Intron 1; black triangles, Int8VNTR and 3'VNTR; horizontal double-arrow, block; inverted open triangle, recombination hotspot for both populations; blue square, genetic selection; large red squares, SUDs-associated polymorphisms. Upper brace, clustering of selections. (B) Distribution of recombination rate across the 18 kb *SLC6A3* promoter regions. Red, AA; blue, EA. *Insert*, a close-up for the region indicated by two arrow heads, by using a finer scale (see x axis). Arrow, transcription start site (TSS). (C) Haploview-based linkage disequilibrium (LD) in *SLC6A3* regulatory regions (18 kb and Int8VNTR). *Left*, AA; *right*, EA. *, block; arrow, location of Int8VNTR. 3'VNTR was not included due to multiple alleles. Parenthesis contains the number of polymorphisms used for the LD analysis. Color: red for stronger LD; white, little LD. Brackets, haplotype blocks defined by Haploview.

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The overall EA LD (D' = 0.9119, r^2 = 0.1876) was bit higher than the AA LD, consistent with the lower recombination rate in EA than in AA. There was a 10 kb block covering from -12499 to -2315, which was located towards the 5' end, approximately 4 kb up compared to the location of the AA weak block (indicated by asterisks in Fig 1C *right panel*). The average LD within this block was D' = 0.9458 and r^2 = 0.2985. Polymorphisms at -5487, -6731, -3182, and -2600 displayed weak LD, D' = 0.5333 and r^2 = 0.06459 on average within this block. There is no LD between -10397G/A (rs6860992) and -4825T/C (rs188332761). Weak LD regions surrounded the transcription start site (TSS), covering the regions from -2296 to +1298 (D' = 0.9507, r^2 = 0.0835). Similar to what was observed in the AAs, the 5' end of the EA Intron 1 also displayed weak LD. Different from the AAs was the 3' end of Intron 1 that displayed strong LD within the region or with upstream regions. Again, Int8VNTR displayed weak LD (D' = 0.6255, r^2 = 0.08734) with upstream polymorphisms. However, Int8VNTR displayed strong LD with -15.0kb-indel, -10250C/T, -1675T/C (rs11564751) and +1787G/A (rs11564757) (D' = 1 and r^2 = 0.4).

When we stratified the 30 subjects by phenotypes, the control LD (D' = 0.8774) was stronger than the case (D' = 0.7992) in the AAs and differences in multifocal pairs were quite significant, by comparing the *upper panels* of S2 Fig. This phenotype-related difference in LD was also observed in EAs, especially for core promoter-Intron 1 *versus* the upstream regions (S2 Fig). Therefore, SUDs-related changes in LD might represent a major difference in *SLC6A3* genetics between AA and EA.

In addition to the biallelic polymorphisms, we also analyzed LD by using the five multiallelic polymorphisms, including -14kb-VNTR, 5'VNTR, -10kb-pA, SSLP and 3'VNTR. Consistently, the AA LD was weaker than the EA LD (S3 Fig *far right panels*). 3'VNTR displayed weak LD with upstream markers: r^2 = 0.049–0.170 in the AAs and 0.115–0.200 in the EAs. Stratification with phenotypes showed that LD in these patients was stronger than in the controls for both populations, based on color intensity (S3 Fig *left panels*).

Genetic selection of polymorphisms

Genetic selection may indicate functionality of polymorphisms. We utilized Tajima's statistic D to evaluate these genetic processes. The average D values for the entire 18 kb promoter were slightly positive (0.48) for EA and slightly negative (-0.29) for AA. However, when we stratified the polymorphisms by variation types, significant D values were revealed. In the AA cohort, VNTRs and SSLP both had significantly positive D values. In particular, the Intron 1 SSLP (heterogeneity or h = 0.7556), 5'VNTR (h = 0.5915) and -10kb-pA (h = 0.6228) in the 5' region had D values of 3.28, 2.67 and 2.48. Between two DNPs, the 5' DNP had a negative D value but the Intron 1 DNP (DNPi) had a positive D (-1.09 and 1.47). The Intron 1 SSLP and DNP were the only types with positive D values in this region. Neither Int8VNTR nor 3'VNTR had significant D values (S4A Fig).

In the EA cohort, all types of variations (-10kb-pA, DNP, VNTR and SSLP) except SNPs had significantly positive D values (2.37, 2.32, 2.45, 4.57 *versus* -0.02857 for SNPs). This EA SSLP displayed the highest heterogeneity of 0.9647 among all of the polymorphisms. The positive DNP D was attributable to both DNPs in the 5' region and in Intron 1. The positive

VNTR D was attributable to 5'VNTR (h = 0.6395) in the 5' region. Unlike the AA D values, SNPs and -15.0kb-indel were the only types that showed negative D values in the EA. The two VNTRs at the 3' side of the gene showed positive but insignificant D values (S4B Fig). It was noticed that SSLP, 5'VNTR and -10kb-pA were all positively selected in both populations.

Haplotypic relatedness

The AA and EA cohorts carried 108 and 79 polymorphisms plus Int8VNTR and 3'VNTR, and these polymorphisms constituted 57 different AA haplotypes (#1–57; #1-#29 from the patients and #30-#57 from the controls; #2, #34 and #40 each occurred twice in the controls) and 57 different EA haplotypes (#58-#114; #58-#87 from the patients and #88-#114 from the controls; #62 and #65 each occurred once in a control and once in a patients and #107 occurred twice in the two controls). That is, three haplotypes each occurred twice and 54 other haplotypes each occurred only once in each cohort of 30 subjects. None of the AA haplotypes were present in the EA and *vice versa*, suggesting that *SLC6A3* carries great diversity not only between different ethnicities but also within the same population.

Co-analyses of the AA and EA haplotypes for relatedness could help understand whether or not the *SLC6A3* haplotypes co-evolved independently between AA and EA during human history. Therefore, we generated a phylogenic tree containing all 114 haplotypes (Fig 2). It turned out that the AA and EA haplotypes were mixed up in terms of relatedness. Overall, the top half of the phylogenetic tree represented a major mosaic of the AA and EA haplotypes, including pairs of 10/61 and 2/68.

Haplotype analysis of three of the EA polymorphisms -14kb-$VNTR_{2/4}$, $Int8VNTR_{5/6}$ and $3'VNTR_{9/10/11}$ showed that 4-6-10 occurred eight times in the patients but did not occur in controls, with a nominal *p* value of 0.0046 by Fisher's Exact Tests (odds ratio (OR) 23.0; 95% confidence interval (CI) 1.26–420.39). The consistencies in allele-specificity of association tendency and the suggestive haplotypic association warrant future investigation of these potential risk factors in large samples.

Recombination hotspots in general populations of European ancestry

To confirm the recombination results from the COGA cohorts, we consulted with the 1KGP and analyzed the entire 70 kb chromosomal region of *SLC6A3*. We combined five European ethnicities, including EA (99 persons), Italy (108 persons), Spain (107 persons), Finland (99 persons), Great Britain (92 persons), for a total of 505 persons. Several hotspots were revealed but the most significant one was in Intron 2 and next to Exon 2. The ones in the core promoter and in Intron 1, revealed by the COGA subjects, were confirmed. Others were located in Introns 4, 7, 8, 11 and 14. None of them were localized to any coding regions. The distal promoter regions were relatively quiet, with six minor ones (Fig 3 *upper part*).

Discussion

It is important to uncover novel, potentially functional polymorphisms and distinct haplotypes, and recombination hotspots because *SLC6A3* activity can be haplotype-dependent partly due to *cis*-antagonism between 5' and 3' sides of *SLC6A3*[95]. The most significant findings from this deep-sequencing study included discovery of novel and selected polymorphisms and the presence of recombination hotspots throughout *SLC6A3*. Although association analysis of SUDs and *SLC6A3* haplotypes was not a primary purpose here, five implications are highlighted as follows.

Fig 2. Unrooted neighbor-joining phylogenetic tree of AA (1–57 in red) and EA (58–114 in blue) regulatory haplotypes of *SLC6A3* (violet, shared branches). 1–29 and 58–87 (labeled in gray bold), carried by the patients and the rest by the controls. For top clade (at least five haplotypes), red arrows, AA clades; blue arrow, EA clade; violet arrow, clades with mixture of two ethnicities. *, carried by two subjects (the rest each carried by one subject). The proportion of sites where at least one unambiguous base was present in at least one haplotype for each descendent clade was either 99% or 100% for each internal node in the tree. The analyses utilized a total of 114 haplotypes.

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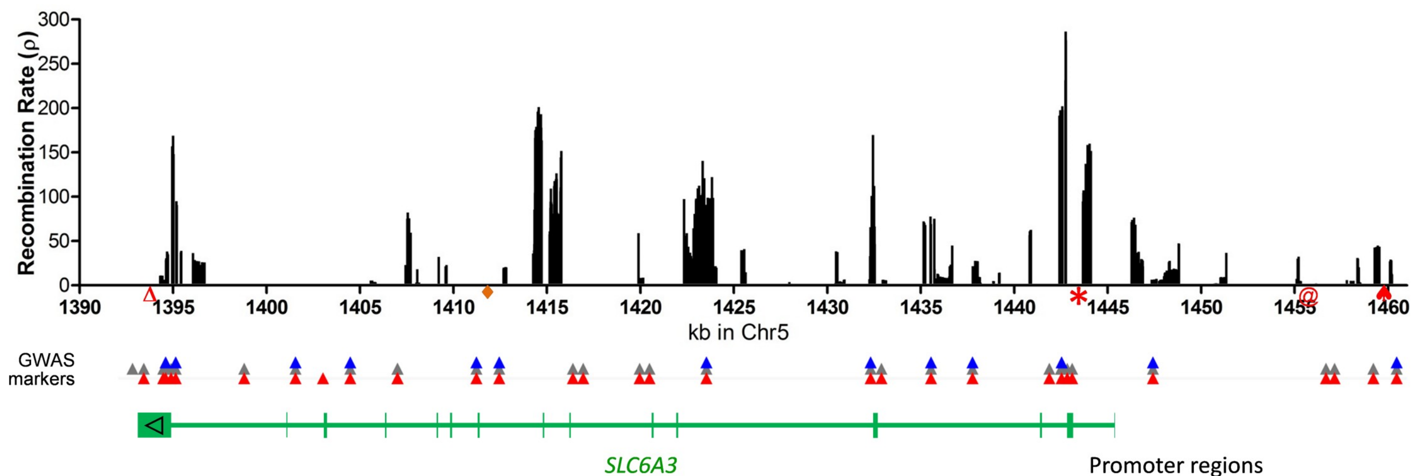

Fig 3. Caucasian recombination hotspots throughout *SLC6A3*. Recombination rate was obtained from combined five European ancestry populations (CEU, TSI, FIN, GBR and IBS for a total of 505 subjects) whose genotype data were collected by the 1000 Genomes Project. Indicated are also two frequently used genotyping markers, 3'VNTR (△), Intron 8 VNTR (◆), DNPi (*), 5'VNTR (@), -14kb-VNTR (♠) and effective GWAS markers (red ▲ for COGA, gray for SAGE and blue for the Australian twin-family study all from dbGaP; "effective" means surviving after the data quality control). Below the GWAS markers is *SLC6A3* gene structure in green (vertical bars are 15 exons in the opposite strand of the chromosome), localizing the polymorphisms and mostly intronic recombination hotspots in the gene. Six populations of African ancestry in the 1KGP (African Caribbean's in Barbados (ACB), Americans in SW USA (ASW), Luhya in Webuye of Kenya (LWK), Esan (ESN) and Yoruba in Ibadan (YRI) of Nigeria, and Mende in Sierra Leone (MSL)) all carried the main hotspots at 1395, 1415, and 1443 (details not shown).

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Great diversity

The levels of variation including novel polymorphisms in the regulatory regions have never been expected. In either of the two cohorts, 60 chromosomes were represented by 57 regulatory haplotypes. The haplotypic diversity was attributable to both high density of polymorphisms and several recombination hotspots. Because of the genetic diversity, the overall LD was generally low, based on the r^2 values of < 0.2 across the regulatory regions and the previously observed balancing selection of promoter haplotypes disappeared here[96, 97]. Such information explains the inconsistent findings on associations between *SLC6A3* and ADHD from genetic studies that used markers located in "random" regions for different populations [98–101]. The rationale of choosing *SLC6A3* markers in most of the studies suffered from the lack of a mechanistic understanding of regulatory genetics in ethnic *SLC6A3* and because of that, the obtained results were more marker- and sample-dependent than phenotype-dependent. Our systemic observations suggest that use of markers that cover more regulatory regions could result in more consistent and positive associations, a notion that has been supported by two studies[42, 46].

Convergence

Phylogenetic analysis indicated for the first time the convergence of the *SLC6A3* regulatory regions between the AAs and the EAs, which means that DNA sequence variation occurred in this promoter independently in the two ethnicities. Despite one main EA clade and three localized minor AA clades, the majority of the haplotypes between two populations were mixed by being localized to many subclades of various sizes. Given the observations that two thirds of the EA polymorphisms were shared by the AA and that the four 5' recombination hotspots were shared between the two ethnicities, it is a reasonable assumption that *SLC6A3* evolves in a largely context-dependent manner. In addition, the mosaic patterns could also be attributable to history of gene flow between European male and African female ancestry [102]. Finally,

this phylogenetic tree indicates a tendency of bidirectional diversion among the 114 18kb-haplotypes. By analyzing twenty six 1KGP populations together (thousands of haplotypes), a much stronger bidirectional diversion of the 18kb promoter was observed (without geographic correlation, data not shown), validating the COGA subjects-based finding and implying again a functional correlation between genetics of the *SLC6A3* promoter and related phenotypes [103].

Ethnicity

As Fig 1C shows, the AAs had clearly overall lower LD than the EAs. Specifically, there were three lines of evidence showing genetic differences in *SLC6A3* between the AAs and the EAs. First, the AA gene had many more common polymorphisms (Fig 1A *upper panel*) than the EA gene (Fig 1A *lower panel*), noticing that most polymorphisms appeared clustered around the recombination hotspots (indicated as inverted triangles). Second, the AA gene lacked polymorphisms with MAF > 0.3 but the EA gene lacked polymorphisms with $0.3 < \text{MAF} < 0.45$ (see gray areas in Fig 1A), suggesting that the AA mutations were less likely to be selected and that the commonest EA variants had been selected. The lack of mutation fixation might have been compensated by the higher mutation frequency in the AA *SLC6A3*. The third evidence was that a haplotype block covered the core promoter region in the AA gene but was larger and covered the center of the 5' region in the EA gene. These genetic differences between the two populations are consistent with the fact that dopamine-related brain function has ethnic differences and suggest that association study needs to use different sets of markers for different populations. These potentially functional polymorphisms might have contributed to the observed genetic selection of particular polymorphisms (see upper braces in Fig 1A).

Selection

Despite the haplotypic diversity, selections of polymorphisms were observed. In both populations, polymorphisms with MAF of 0.31–0.40 were de-selected. For the most common or ethnicity-specific polymorphisms (MAF of > 0.40), de-selection was the most significant in the AAs whereas selection was the most significant in the EAs, representing the largest difference between the two ethnicities. Tajima's D statistic has been used in trying to identify functional polymorphisms[104, 105] and indicates here the selection of some polymorphisms in both cohorts. We now report indeed that Tajima's D results are consistent with the functionality of DNPi because the EA DNPi was positively selected and this DNPi indeed mediates a long non-coding RNA (lncRNA) regulation of the *SLC6A3* promoter[75]. Significantly, SSLP in Intron 1, 5'VNTR and -10kb-pA were all selected in both populations, consistent with the fact that *SLC6A3 cis*-acting elements such as the 5 kb super enhancer (5KSE) can be localized to distal 5' regions[95] and that 5'VNTR was positively correlated with the mRNA levels in postmortem dopamine neurons[76]. These positive selections thus generate testable hypotheses for functionality and associations.

Localized recombination

SLC6A3 is located at 1.4 Mb, near a telomere of chr5, and harbors several hotspots, consistent with previous observations that regions near telomeres tend to have higher recombination rates than those near centromeres[106–109]. The identified recombination hotspots not only help explain negative findings on *SLC6A3* in previous GWAS but also guide selection of new association markers. Fig 3 *lower part* indicates genetic markers used by three previous GWAS, in reference to the distribution of the recombination hotspots. It is noticed that all of these markers are separated from the core promoter by recombination hotspots. The two widely

used VNTRs, located in last exon and Intron 8, are each separated from the promoter regions (also as previously suggested [110]) by several recombination hotspots, suggesting that these markers are unable to capture information effectively on varying promoter activity[51]. As such, the previous association studies have not effectively used genetic markers for this gene yet. The recently identified functional DNPi (indicated by an asterisk)[75] is buried in two hotspots, suggesting that none of the previously used markers could capture the DNPi-related information. We have tried to use either the 1KGP or the COGA genotype as the templates to impute DNPi in the other GWAS datasets but failed completely. This is partly explained by the fact that DNPi has low LD with other markers and is located in a small, isolated haplotype block (S5 Fig). When sample sizes get large enough and using the 1KGP with strong LD as the template (S5 Fig), DNPi could be imputed but the results would not be reliable for cohorts of interest[111]. Therefore, this marker should be experimentally typed for accurate association information.

We acknowledge that the main limitations of this study include small sample sizes which aimed at common variants only, limited number of ethnicities and mainly the promoter regions. Including the patients might affect any ethnic comparisons and LD might cause overestimation of distances in the phylogenetic tree. The results, however, should provide guidance for rational selection of genetic markers for functional and association studies, as we have done before[51, 76], and for in-depth interrogations of the entire gene in more ethnicities.

Conclusions

Extensive DNA sequence variations not only around the core promoter but also in other distal regulatory regions may work in concert or in haplotypes and influence dopamine-related individuality and diseases. Such genetic diversity of *SLC6A3* may help explain the elusiveness of previous association findings with the classical markers in the 3' side. The findings also lay a foundation for a better understanding of the roles that the polymorphic *SLC6A3* plays in human brain.

Supporting information

S1 Fig. Details of multiple alleles of -14kb-VNTR (*upper panel*) and Intron 1 SSLP (*lower panel*).
(PDF)

S2 Fig. Haploview-based linkage disequilibrium (LD) in *SLC6A3* regulatory regions by phenotypes of the two COGA cohorts.
(PDF)

S3 Fig. SHEsis-based LD by multi-allelic polymorphisms in the COGA cohorts.
(PDF)

S4 Fig. Genetic selection of polymorphisms by Tajima's statistic D for AA (a) and EA (b).
(PDF)

S5 Fig. Significant difference in 18kb *SLC6A3* promoter LD (D') between 1KGP CEU (upper) and COGA sample (30 controls and 30 patients with SUDs).
(PDF)

S1 Table. PCR primers used.
(XLSX)

S2 Table. Common and unique alleles in COGA samples.
(PDF)

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